보건학석사 학위논문

Screening thyroid hormone disrupting effects of benzophenones using GH3, FRTL-5 cells and zebrafish

GH3, FRTL-5 세포주와 제브라피쉬를 활용한

벤조페논류의 갑상선 교란 영향 스크리닝

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서울대학교 보건대학원

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이 정 은

Abstract

Screening thyroid hormone disrupting effects of benzophenones using GH3, FRTL-5 cells and zebrafish

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Benzophenones (BPs) are UV protection agents frequently used in various personal care products (PCPs). BPs have been widely detected in the environment and biota. Endocrine disrupting effects of some BPs have been documented. However, significant knowledge gaps are present for thyroid disrupting effects of these compounds.

Thyroid disruption of various BPs was investigated using rat pituitary and thyroid follicle cell lines, and zebrafish. First, *in vitro* assays employing a rat pituitary cell line (GH3) and a rat follicular cell line were conducted on six BPs, i.e., benzophenone (BP), benzophenone-1 (BP-1), benzophenone-2 (BP-2), benzophenone-3 (BP-3), benzophenone-4 (BP-4), and benzophenone-8 (BP-8). Then, BP-3, mainly used BP-type UV filter, and its major metabolites BP-1 and -8 were employed for subsequent *in vivo* tests with zebrafish (*Danio*

rerio) embryo.

Following *in vitro* GH3 exposure, all six BPs except BP-4 down-regulated *Tshβ*, *Trhr*, and *Trβ* genes and up-regulated *Dio2* gene in the rat pituitary cells. In addition, some BPs significantly up-regulated *Nis* and *Tg* genes while down-regulating *Tpo* gene on various level in FRTL-5 cells. In zebrafish embryo, significantly decreases of whole-body T4 and T3 level were observed following exposure to each BP until 144 hour post fertilization (hpf). BP-3 and -8 decreased T3 in zebrafish at lower exposure concentrations compared to that for BP-1, implying greater thyroid hormone disrupting potencies of both BPs. Transcriptional changes in several thyroid hormone regulating genes in hypothalamic-pituitary-thyroid axis were observed as well.

The results of this study showed that all tested BPs caused thyroid disrupting responses in a rat pituitary gland and a thyroid gland, crucial organs regulating homeostasis of thyroid system. Embryo-larval exposure of zebrafish also demonstrated that BP-1, -3, and -8 could alter thyroid hormone levels. Since thyroid hormone regulation plays key role in early development and normal physiology, consequences of this thyroid hormone disruption in later life stages of the fish warrant further investigation.

Key words: benzophenones, UV-filter, GH3 cell line, FRTL-5 cell line, zebrafish, thyroid hormone, endocrine disruption, screening

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1. Introduction

Benzophenones (BPs) are frequently used as UV protection agents in personal care products (PCPs) such as sunscreen, nail polish, lipsticks, shampoo, and hand sanitizer. As amount of PCPs use has increased, their detection in the environment and biota have been increasingly reported. BPs are widely detected in the environmental media such as surface water and wastewater around the world (Tsui et al 2014; Ramos et al., 2016; Montes-Grajales et al., 2017). In addition, these compounds were found in human specimens such as urine, serum, breast milk, adipose tissue, and placental tissues (Gao et al., 2015; Hines et al., 2015).

BPs have been recognized for endocrine disrupting effects, especially related to sex hormones (Kunz et al., 2006; Blüthgen et al., 2012; Kim et al., 2014). BP-3, mainly used BP-type UV filter, showed estrogenic effects in Japanese medaka (*Orizias latipes*) and rats, which was attributed to estrogenic effects of BP-1, a major metabolite of BP-3 (Kim et al., 2014; Schlumpf et al., 2001; Schlecht et al., 2004; Suzuki et al., 2005). As BP-3 could be metabolized into other BPs, i.e. BP-1 or -8, and each BP has been also used as UV protection agents in PCPs, more toxicological information is necessary for BPs other than BP-3. Meanwhile, endocrine disrupting effects of BPs have concentrated on sex hormone disruption, and thyroid hormone disrupting effects of this group of chemicals are not well understood to date.

Thyroid hormones play crucial role in organisms for development, growth and energy metabolism. Therefore, disruption or alteration in thyroid system would cause various types of adverse effects to organisms (Yen et al., 2001;

Patrick et al., 2009). Thyroid hormone system is hence tightly regulated by feedback system based on circulatory and peripheral levels of thyroid hormone, thyroxine (T4) and triiodothyronine (T3). The thyroid hormones are synthesized in thyroid gland activated by thyroid-stimulating hormone (TSH) from pituitary gland, and several proteins like sodium iodide transporter (NIS), thyroid peroxidase (TPO) are involved in this process. Most of thyroid hormones in circulation of organism exist as a form of T4, an inactive form, and this is converted into an active T3 by deiodinases in peripheral tissues. In liver, excess amount of thyroid hormone is excreted by UGT enzyme. Many environmental chemicals have been suggested to disrupt the regulations of synthesis, transportation, deiodinase activity, or and hepatic metabolism of thyroid hormones (Boas et al., 2006; Miller et al., 2009).

BP-2 is one of the best-known thyroid disruptors in both *in vitro* and *in vivo* studies (Jarry et al., 2004; Schmutzler et al, 2007a). BP-2 decrease serum T4 and T3 level in rats (Jarry et al., 2004; Schmutzler et al., 2007a). Thyroid peroxidase (TPO) involved in thyroid hormone synthesis is known to be inhibited by exposure to BP-2 and this could lead to decrease in thyroid hormone level (Schmutzler et al., 2007a; Miller et al., 2009; Krause et al., 2012). However, studies on thyroid disruption of BPs have mostly been limited to BP-2 and TPO inhibition.

There are increasing reports suggesting thyroid hormone disrupting potencies of other BPs: Human observations reported negative associations between urinary BP-3 and serum T4 or T3 (Aker et al., 2016; Kim et al., 2017). Therefore, significant knowledge gaps are present for thyroid hormone

disrupting effects of other structural analogues of BP-2.

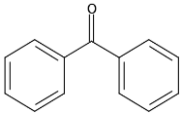
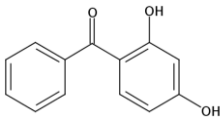
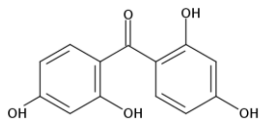
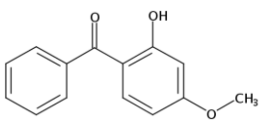
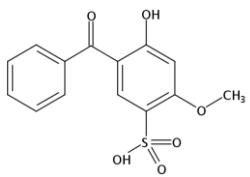
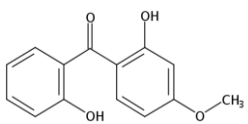
The object of this study is to evaluate thyroid disrupting effects of BPs. This study was designed in two steps. First, thyroid disrupting potencies of BPs were compared using a rat pituitary cell line (GH3) and a rat thyroid follicular cell line (FRTL-5). Based on these *in vitro* assays, three potent BPs were chosen and applied to *in vivo* studies employing zebrafish larvae. In fish, the effects on hypothalamic-pituitary gland-thyroid axis and thyroid hormones were evaluated. The results of this study will help identify BPs with thyroid disruption effects, and understand associated mechanisms.

2. Materials and Methods

2.1 Chemicals

Tested BPs, i.e., BP (benzophenone, CAS no. 119-61-9, ≥99%), BP-1 (2,4-dihydroxybenzophenone, CAS no. 131-56-6, 99%), BP-2 (2,2′,4,4′-tetrahydroxybenzophenone, CAS no. 131-55-5, 97%), BP-3 (2-hydroxy-4-methoxybenzophenone, CAS no. 131-57-7, 98%), BP-4 (2-hydroxy-4-methoxybenzophenone-5-sulfonic acid, CAS no. 4065-45-6, ≥97%), and BP-8 (2,2′-dihydroxy-4-methoxybenzophenone, CAS no. 131-53-3, 98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The physicochemical properties are shown in Table 1. T3 (Triiodothyronine, CAS no. 6893-02-3) and TSH (Thyroid-stimulating hormone, CAS no. 9002-71-5), positive chemicals, were purchased from Sigma-Aldrich. As a solvent, dimethyl sulfoxide (DMSO, CAS no. 67-68-5) was used. Hybri-Max^{TM} grade DMSO (purity≥99.7%) was purchased from Sigma-Aldrich, and for fish exposure DMSO with purity≥99% was purchased form Junsei Chemical Co. (Tokyo, Japan).

Table 1. Molecular structure and characteristics of tested benzophenones

	BP	**BP-1**	**BP-2**
Structure			
CAS RN	119-61-9	131-56-6	131-55-5
MW (g/mol)	182.22	214.22	246.22
LogKow[a]	3.214±0.291	3.152±0.360	3.091±0.419
	BP-3	**BP-4**	**BP-8**
Structure			
CAS RN	131-57-7	4065-45-6	131-53-3
MW (g/mol)	228.24	308.31	244.24
LogKow	3.995±0.366	0.993±0.393	4.311±0.409

[a]LogKow value was obtained from SciFinder (https://scifinder.cas.org/).

2.2 GH3 cell culture and exposure

The GH3 cell line was obtained from Korean Cell Line Bank (Seoul, Korea) and was maintained at 37 ◦C with 5% CO_2. The cells were grown in a Dulbecco's modified Eagle's medium/Ham's F-12 nutrient mixture (Sigma–Aldrich), supplemented with 10% fetal bovine serum (FBS; Gibco®, LifeTechnologies, Carlsbad, CA, USA) as performed in Kim et al. (2015).

For exposure, the GH3 cells were seeded in 24-well plates at a density of 2.5×10^5 cells/well and then incubated for 20 h. To get rid of the effects from steroid hormones and growth factors in serum (FBS), the growth medium was changed into serum-free medium 4-hour-before exposure which contains 1% BD ITS^+ premix (BD Biosciences, Franklin Lakes, NJ, USA) and then incubated for further 4 h.

The cells were exposed to different dose range of BPs and T3. Test dose ranges were determined at non-cytotoxic levels based on preliminary cytotoxicity assay performed with WST-1 cell proliferation assay (Roche Applied Science, Mannheim, Germany). The test concentration was as follows: 0, 3.2, 10, 32 or 100 μM for BP; 0, 1, 3.2, 10 or 32 μM for BP-1; 0, 0.32, 1, 3.2 or 10 μM for BP-2; 0, 3.2, 10, 32 or 100 μM for BP -3; 0, 32, 100 or 320 μM for BP-4; 0, 3.2, 10, 32 or 100 μM for BP-8.

The test doses were in triplicate (n=3) for each treatment (0.1% v/v DMSO). T3 was used as a positive control at 1 nM and treated in each set of exposure for verification. Following 48 h exposure, cells were washed with phosphate-buffered saline (PBS) and extracted for RNA.

2.3 FRTL-5 cell culture and exposure

FRTL-5 cells were maintained at 37 ℃ in a 5% CO_2 atmosphere in Coon's modified Ham's F-12 medium (Sigma-Aldrich) supplemented with 10% calf serum (Gibco®) and a mixture of 6 hormones (6H medium): insulin (1 μg/mL), transferrin (5 μg/mL), somatostatin (10 ng/mL), gly-his-lys acetate (10 ng/mL), hydrocortisone (10 nM), and thyroid stimulating hormone (TSH, 1 mU/mL) following a previous study (Kim et al., 2015). All the hormones used in the medium were purchased from Sigma–Aldrich. The 6H medium was supplemented with L-glutamine (2 mM; Gibco®) and MEM non-essential amino acids (1 mM; Gibco®). FRTL-5 cells in density of 8.0 × 104 cells/well were seeded in 24-well plates and incubated for 24 h with 6H medium. After 24 h, the medium was exchanged to 5H medium (6H medium without TSH) and incubated for 24 h. The cells were then dosed with a series of concentrations of each chemical: 0, 10, 32, 100, or 320 μM for BP, BP-1, -2, -3, or -4 and 0, 3.2, 10, 32 or 100 μM for BP-8. These experimental doses were determined based on preliminary range-finding tests at non-cytotoxic doses (Supplementary information, Fig. S2). TSH (10 mU/mL) was used as a positive control (Kim et al., 2015). The cells were exposed to each chemical for 24 h in three technical replicates (n = 3) with three biological replicates.

2.4 Zebrafish culture and embryo/larval exposure

Adult zebrafish were raised in Environmental Toxicology Laboratory, SNU (Seoul, Korea). Fertilized eggs were obtained by mating sexually mature adult fish. Embryos were randomly divided into 500 mL glass beakers which

contain 300 mL exposure media, within 5 hours after fertilization.

Based on preliminary range finding test, the exposure concentrations for each chemicals were determined at 0, 100, 320, and 1000 μg/L for BP-1; 0, 32, 100, and 320 μg/L for BP-3; 0, 32, 100, and 320 μg/L for BP-8. The exposure media was made with dechlorinated water with DMSO stock (0.005% v/v) and was replaced daily until 6 day post-fertilization of exposure.

During the exposure, the embryos were raised around 26±1 ◦C with a 14 L:10 D photoperiod. Water quality parameters such as conductivity, temperature, pH and dissolved oxygen, were measured regularly after renewal of exposure media.

The embryo and larval survival, hatchability were observed daily. At 6 dpf 20 larvae were randomly sampled for gene analysis and another 180 larvae were employed for thyroid hormone measurement. After collection, larval weight was measured and the fish were stored at −80 ◦C until being used for analysis.

2.5 Thyroid hormone extractions and measurement

For thyroid hormone extraction, 180 zebrafish larvae per replicate were homogenized in 110 μL ELISA buffer, using a motor driven tissue grinder (Ginbko Bioscience, China). The homogenates were sonicated for 10 min at 4 ◦C and centrifuged at 5000×g for further 10 min at 4 ◦C. The supernatant was collected and stored at −80 ◦C until analysis.

T4 and T3 levels were measured using enzyme linked immunosorbent assays (ELISA) as performed in Yu et al. (2010), with minor modification. The test kits (Cat no. CEA452Ge for T4; Cat no. CEA453Ge for T3) were purchased

from Cloud-Clone Corp. (Wuhan, China), and the detection limits for T4 and T3 were 1.42 ng/mL and 47.2 pg/mL each. Measurement was conducted by Tecan Infinite® 200 (Tecan Group Ltd., Mändorf, Switzerland) following manufacturer's instructions.

2.6 RNA isolation and quantitative real-time polymerase chains reaction (qRT-PCR)

For RNA isolation in GH3 assay, the cells were washed twice with ice-cold PBS and lysed with lysis buffer. And in zebrafish larvae experiment, 20 larvae were grinded in lysis buffer with tissue grinder. RNA was immediately isolated using RNeasy mini kit (Qiagen, Hilden, Germany) in each experiment. The RNA quality and concentration were measured with a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA). After dilution of mRNA to make the same concentration in each sample, complementary DNAs (cDNAs) were synthesized using the iScriptTM cDNA synthesis kits (BioRad Hercules, CA, USA).

For quantitative real-time PCR (qRT-PCR), the 20 μL of qRT-PCR mix reaction mix was prepared with 10 μL of LightCycler® 480 SYBR Green I Master mix (Roche Diagnostics Ltd., Lewes, UK), 1.0 μL of each PCR primer (10 pmol), 6 μL of purified PCR-grade water, and 2 μL of the cDNA diluted 1:4 with water. The primer sequences used in this study are as shown in supplementary information, Table S1. *Gapdh*, *β-actin* and *18s* genes were used as housekeeping genes in GH3, FRTL-5 cell assays and zebrafish larvae gene analysis, respectively. qRT-PCR was performed using LightCycler 480

(Roche Applied Science, Indianapolis, IN, USA). The thermal cycle profile was: pre-incubation at 95 ◦C for 10 min, 40 cycles of amplification at 95 ◦C for 10 s, 85 ◦C for 20 s, and 72 ◦C for 20 s.

For quantification of the PCR results, the threshold cycle (Ct) was determined for each reaction. The Ct values for each gene of interest were normalized to the housekeeping gene using the $2^{-\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.7 Statistical analysis

The data normality and homogeneity of variances were analyzed by Shapiro–Wilk's test and Levene's test, respectively. One-way analysis of variance (ANOVA) with Dunnett's test was used for comparison among control and treatments. The p-value < 0.05 was considered significant. Linear regression analysis was conducted for trend analysis and the slope and significance of the linear trend were shown when p-value was less than 0.05. Mean value was expressed with standard error of the mean (SEM) in all data. IBM SPSS 20.0 for Windows (SPSS Inc., Chicago, IL, USA) was used in data analysis.

3. Results

3.1 Transcriptional changes related to thyroid system in GH3 cells

Exposure level of T3, a positive chemical for GH3 assay, was determined at 1 nM based on Kim et al. (2015). The responses of T3 exposure in the present study were similar to those reported in Kim et al. (2015): Significant down-regulation of *Tshβ*, *Trhr*, and *Trβ* genes and up-regulation of *Dio2* gene were observed.

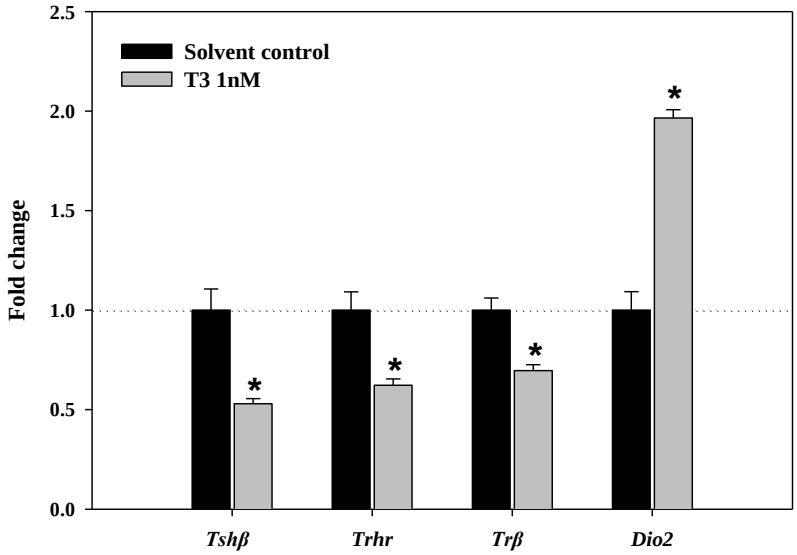


Figure 1. Transcriptional changes related to thyroid system by exposure to a positive control (T3) in GH3 cells. The relative expression level after T3 exposure was indicated in means±SEM (n=3). Asterisks denote significant differences from solvent control ($p<0.05$).

The responses of GH3 cells following exposure to BPs were in similar direction of those obtained from T3 exposure. Transcriptional level of *Tshβ* gene following exposure to all BPs decreased to ≤ 0.75 fold compared to the solvent control, and all transcriptional changes were statistically significant except for BP-4. Similarly, in case of *Trhr*, they showed down-regulation to ≤ 0.75 fold except BP-4. In addition, BPs did not suppress *Trβ* transcription: only BP-3 showed significant decrease in transcription. In case of *Dio2*, we observed significant up-regulation in case of BP, BP-3 and BP-8. At the same time BP-2 also showed highly up-regulated transcriptional level (>1.5 fold) although statistical significance was not observed.

Among six BPs, BP-1, -2, -3 and -8 showed greater potencies in transcriptional alteration of the thyroid regulating genes. As thyroid disruption potential and associated mechanisms are relatively well-known for BP-2, only BP-1, -3, and -8 were chosen and employed subsequently for zebrafish exposure.

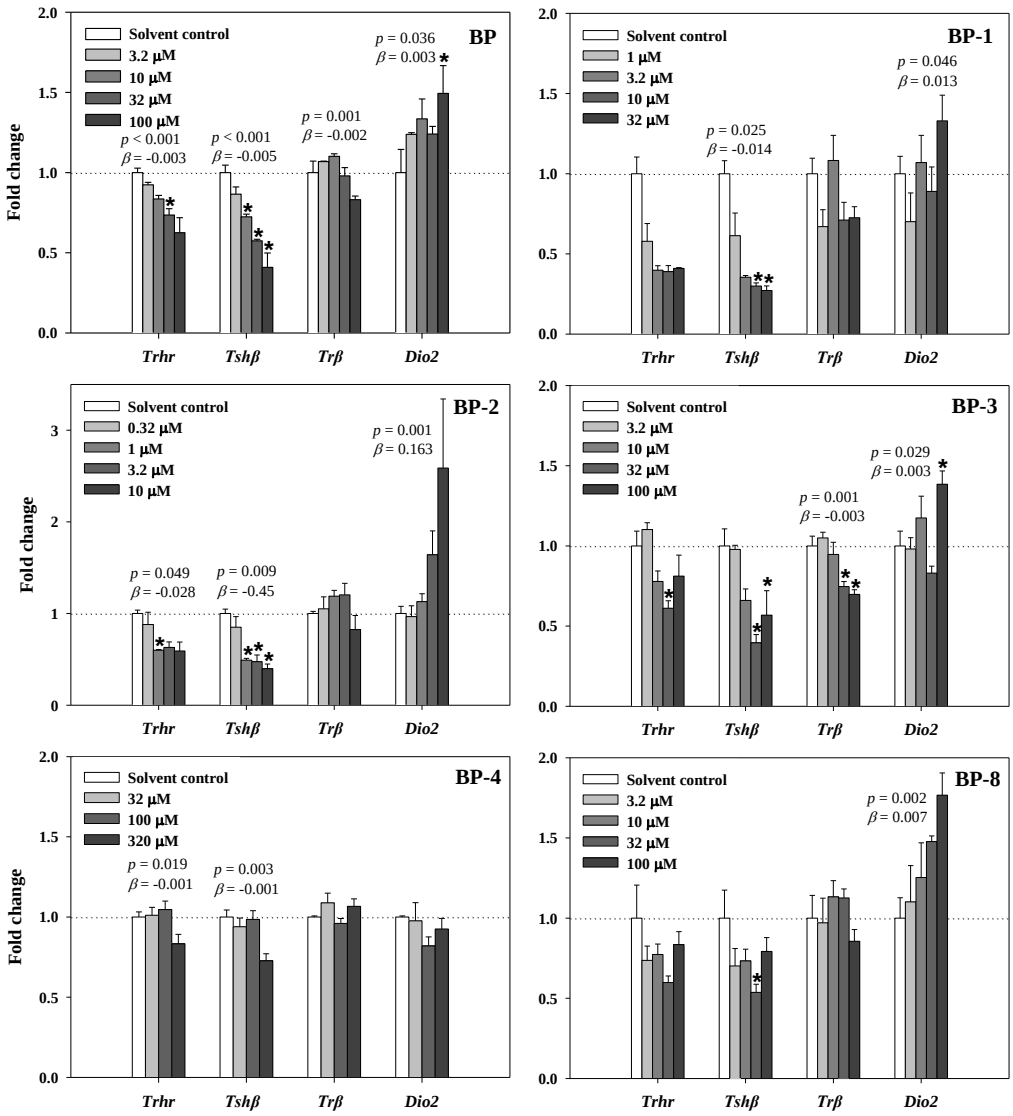


Figure 2. Transcriptional changes related to thyroid system by exposure to tested benzophenones (BP, BP-1, -2, -3, -4, and -8) in GH3 cells. The relative mRNA expression level was shown as means±SEM (n=3). Asterisks denote significant differences from solvent control (p<0.05). The p-value and β value shown indicate p and slope for trends, respectively, for the given transript.

3.2 Transcriptional changes related to thyroid system in FRTL-5 cells

TSH was exposed at 10 mU/mL as a positive chemical for FRTL-5 assay (Kim et al., 2015). Significant up-regulation in *Nis* gene by 9.6-fold and down-regulation in *Tshr*, *Tpo*, and *Tg* genes were observed by exposure to TSH (Fig. 3).

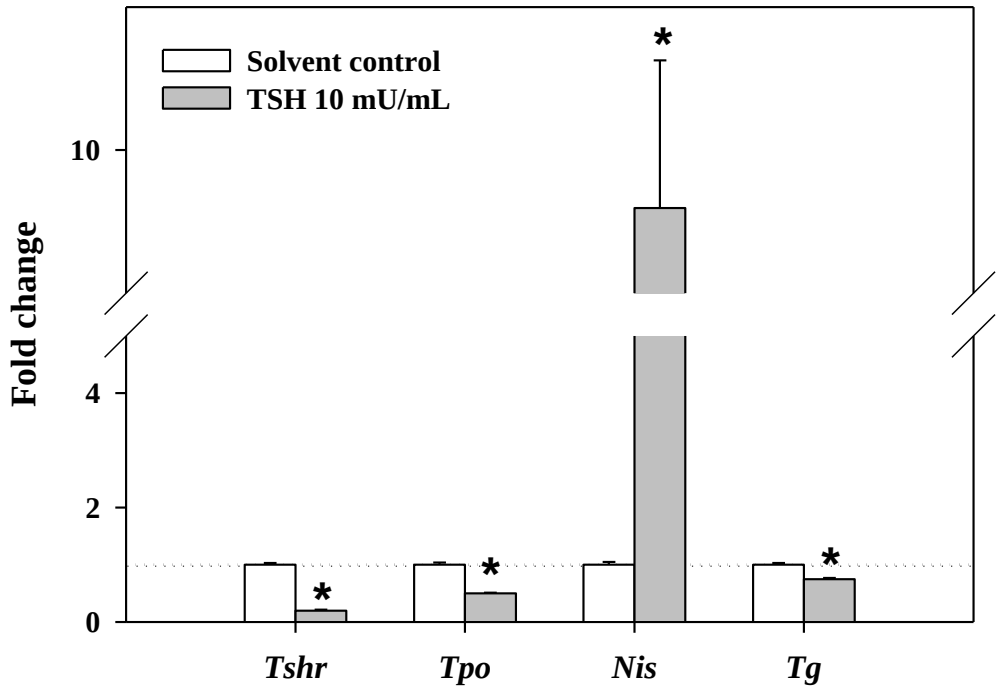


Figure 3. Transcriptional changes related to thyroid system by exposure to a positive control (TSH) in FRTL-5 cells. The relative expression level after TSH exposure was indicated in means±SEM (n=3). Asterisks denote significant difference from solvent control ($p<0.05$).

Significant down-regulation in *Tpo* gene was observed following exposure to BP-1, -2, -3, and -8 in FRTL-5 cells. BP-8 down-regulated expression level of *Tpo* gene to < 0.5-fold even at 100 μM, but for BP-1 and -2, transcriptional changes to below 0.5-fold were observed at 320 μM. In addition, all tested BPs except BP-4 up-regulated transcriptional level by over 1.5-fold with greater potencies in BP-1, -3 and -8. *Tg* gene was also up-regulated by over 1.5-fold following exposure to BP, while exposure to BP-1 and -8 highly stimulated *Tg* gene by over 3.0-fold. However, all BPs did not affect *Tshr* gene by exposure.

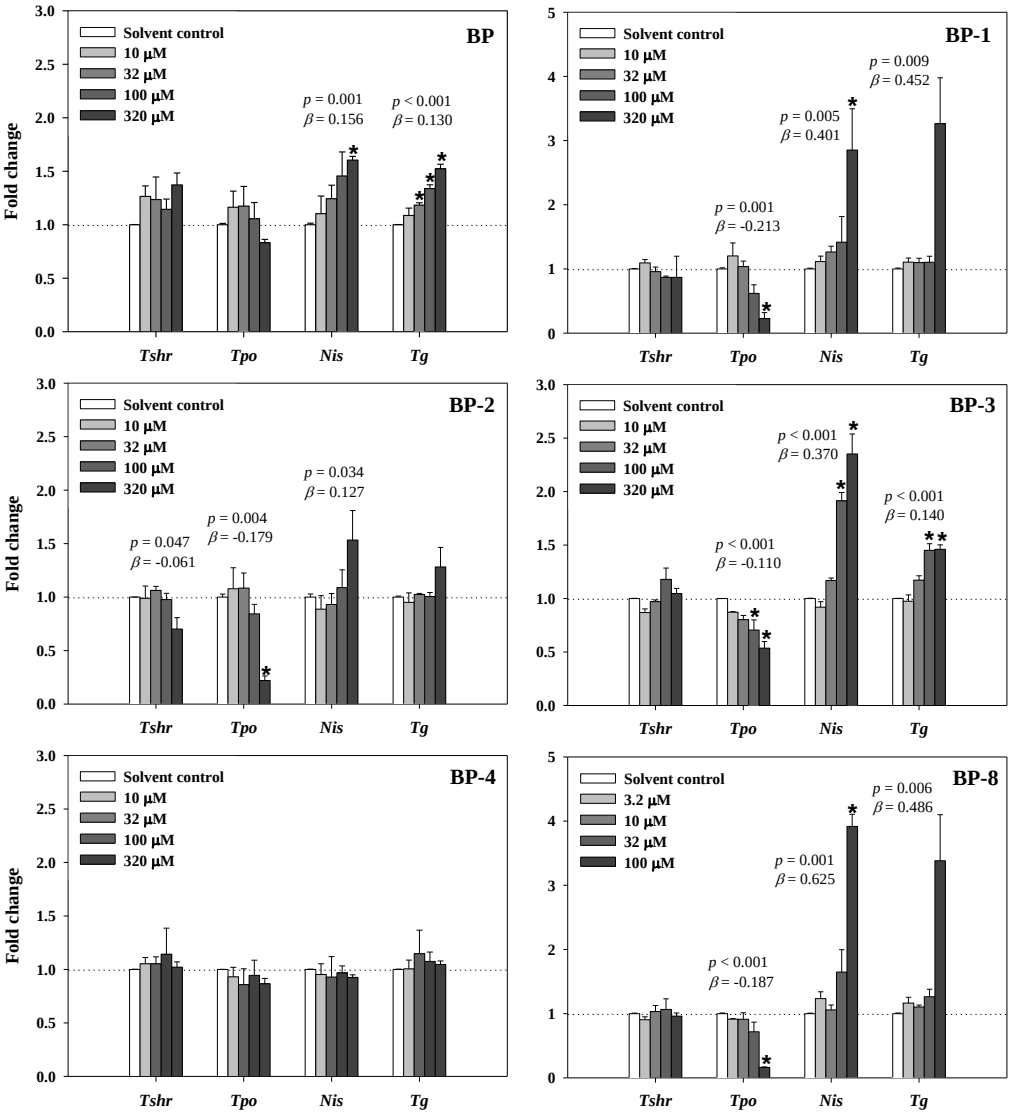


Figure 4. Transcriptional changes related to thyroid system by exposure to tested benzophenones (BP, BP-1, -2, -3, -4, and -8) in FRTL-5 cells. The relative mRNA expression level was shown as means±SEM (n=3). Asterisks denote significant difference from solvent control (p<0.05). The p-value and β value upper side of the bar indicate p and slope for trends, respectively.

3.3 Effects on thyroid hormone level in zebrafish

Exposure to BP-1, -3, or -8 until 144 hpf significantly decreased whole-body T4 and T3 levels in zebrafish larvae (Fig. 5). BP-1 showed significant T4 and T3 decrease at 320 and 1000 μg/L group, but BP-3 and -8 showed significant changes in T3 level at all treatmentss, but not in T4 level. BP-3 and BP-8 caused T3 decrease generally at lower exposure doses compared to BP-1, while only BP-1 significantly decreased T4 level in zebrafish.

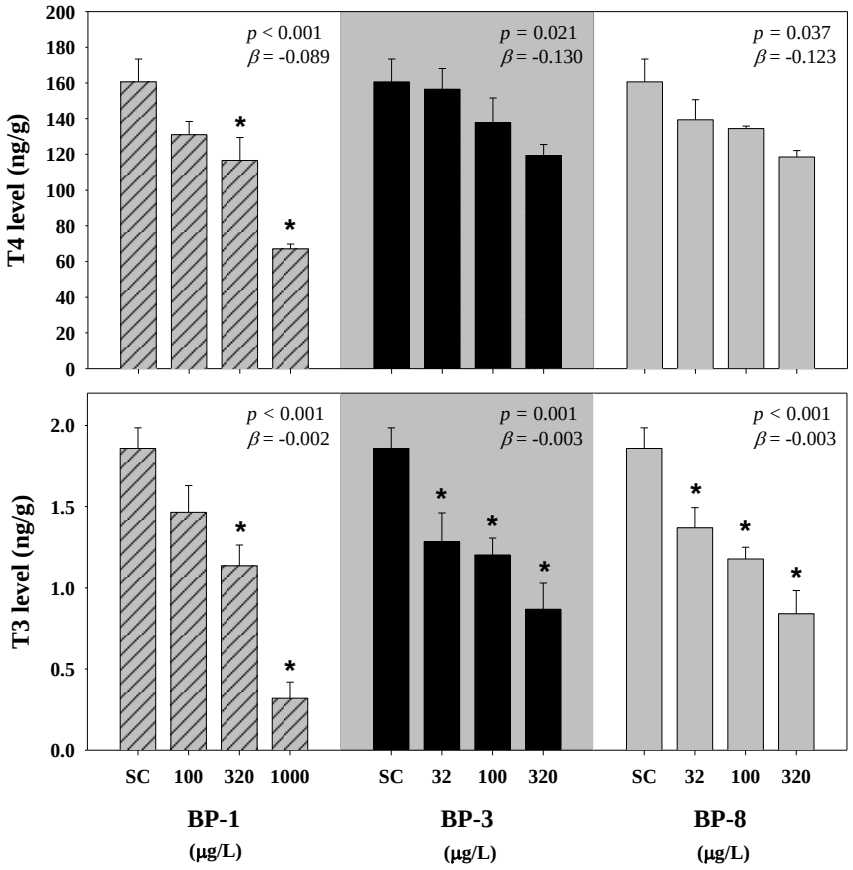


Figure 5. T4 and T3 levels in whole-body of 144 hpf zebrafish larvae by exposure to BP-1, -3, or -8 (n=4). The T4 and T3 level are shown as mean±SEM (n= 4). Asterisks denote significant difference from solvent control (p<0.05). The p-value and β value shown indicate p and slope for trends, respectively, for a given hormone.

3.4 Transcriptional changes related to thyroid system in zebrafish

Significant up-regulation of thyroid related genes was observed after exposure to BP-3 or BP-8, but not by BP-1 exposure (Fig. 6). BP-3 significantly up-regulated *tg*, *dio1*, and *ugt1ab* genes and BP-8 significantly up-regulated all genes except *tshβ* in 100 μg/L group. Although not all of them showed statistical significance, all genes analyzed in this study was up-regulated by >1.5 fold after exposure to BP-3 and BP-8. Especially in case of BP-3, all measured genes had significantly increasing trend (p-value < 0.05, trends are not shown). Although we observed significant changes in thyroid hormone level after exposure to BP-1 to zebrafish, there was no significant change in thyroid hormone related genes in BP-1 exposure group.

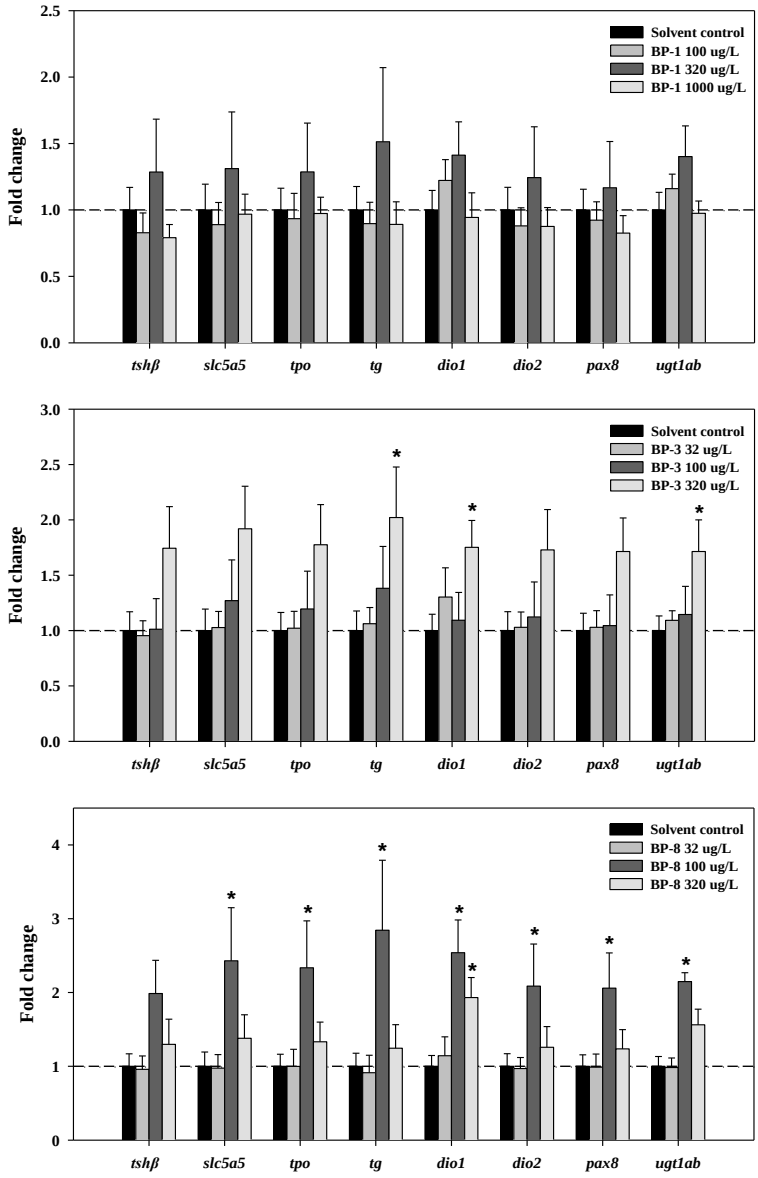


Figure 6. Transcriptional changes related to thyroid system by exposure to BP-1, -3, or -8 in whole-body of 144 hpf zebrafish larvae. The relative mRNA expression levels are shown as mean±SEM (n=4). Asterisks denote significant difference from solvent control (p<0.05)

3.5 Effects on survival, hatchability and body weight of zebrafish

Following exposure to BP-1, -3, or -8 of zebrafish until 144 hpf, the embryo survival and larval survival, hatchability and body weight of zebrafish were not affected (Table 2). In case of BP-8 320 μg/L group, there was one replicate which showed a notable decrease in hatchability (67%). However, this response was significantly different from those observed from the other replicates of the given treatment. Therefore, this replicate was excluded from further analysis, assuming it as an outlier caused by factors not directly related to the experimental design (n=3).

Table 2. Effects of BP-1, -3, or -8 exposure on embryo/larval survival, hatchability and body weight in zebrafish

Chemicals (μg/L)		**Embryo survival[a] (%)**	**Larval survival[b] (%)**	**Hatchability[c] (%)**	**Body weight[d] (mg)**
Solvent control		94.9±1.3	98.3±0.3	88.9±1.3	63.0±1.2
BP-1	100	97.3±1.0	98.2±0.2	89.3±0.9	61.7±2.7
	320	95.9±0.8	98.2±0.3	87.6±0.9	59.0±1.6
	1000	96.4±1.2	97.6±0.4	82.4±1.5	58.8±0.4
BP-3	32	94.9±1.5	98.4±0.4	87.0±1.9	62.7±2.5
	100	98.0±0.8	98.9±0.2	90.3±0.8	64.1±1.3
	320	98.4±0.3	98.2±0.3	91.4±1.2	61.8±1.0
BP-8	32	97.3±1.1	97.9±0.5	89.0±2.4	59.6±1.5
	100	89.8±2.5	97.3±1.2	82.1±1.9	63.4±2.7
	320	97.1±1.7	97.9±0.3	81.2±4.2	60.7±2.3

[a]Embryo survival is shown as percentage of surviving embryos among the fertilized eggs. [b]Larval survival is shown as percentage of surviving larvae among the all hatched larvae. [c]Hatchability is shown as percentage of the hatched larvae among all the fertilized embryos. Dead eggs were counted as non-hatched. [d]Body weight was measured as wet weight of 180 zebrafish larvae. Values are mean±SEM (n=4, except for BP-8 320 μg/L which was n=3).

4. Discussion

The responses of a rat pituitary cell (GH3 cell) and a rat thyroid gland cell (FRTL-5 cell) following exposure to each of six BPs (Figs. 2 and 4) clearly show that the tested BPs affect hypothalamic-pituitary-thyroid (HPT) axis and have thyroid disrupting effects. Changes in thyroid hormone levels in zebrafish larvae following exposure to three BPs, i.e. BP-1, -3 and -8 (Fig. 5), also confirm thyroid disrupting effects of these widely used consumer chemicals.

In GH3 cell assays, all tested BPs showed the same directions of transcriptional changes in key thyroid hormone regulating genes, but with different potencies (Fig. 2). Down-regulations in *Trhr* and *Tshβ* genes by BPs were similar to the responses of GH3 cells following exposure to T3, which suggest that BPs may act in similar way with T3 on a pituitary gland (Fig. 1). In this process, decrease in thyrotropin-releasing hormone (TRH) receptor, encoded by *Trhr*, could reduce signals from TRH. As TRH stimulates pituitary gland to release TSH, subsequent repressed translation of *Tshβ* into TSH could lead to decrease in thyroid hormone level (Fig. 5). There are several environmental chemicals have been reported to disrupt central regulation of thyroid system such as TRH or TSH receptor signaling (Murk et al., 2013). Our observation shows that six BPs can also disrupt central regulations of thyroid hormones homeostasis.

Down-regulation in *Trβ* gene was observed after exposure to BP-3 and similar trends being observed for most of the other BPs (Fig. 2) suggest that

BPs could directly down-regulate thyroid hormone receptor to inhibit interaction with thyroid hormone. Thyroid hormone acts through thyroid hormone receptor. The *Trβ* gene is mainly expressed in pituitary gland and T4 could bind to this receptor more effectively after being activated into T3 by *Dio2* leading to negative feedback action of thyroid hormone (Gutleb et al., 2005; Zoller et al., 2007). Significant up-regulation in *Dio2* gene by BP, BP-3 or -8 (Fig. 2) suggests that BPs may activate conversion into T3 partially in a pituitary gland, which may affect synthesis of thyroid hormones in thyroid gland.

In FRTL-5, each BPs down-regulated *Tpo* gene on various levels, which indicates they can act in similar way each other as previously reported on BP-2 (Fig. 4). TPO is an enzyme involved in iodine organification, i.e., coupling of iodide to thyroglobulin (Zoller et al., 2007). In previous studies, BP-2 decreases thyroid hormone through inhibiting TPO activities (Jarry et al., 2004; Paul et al., 2014; Schmutzler et al, 2007a). In Amplex UltraRed-TPO inhibition assay, BP-2 disrupted TPO activity while BP-3 did not show any change (Paul et al., 2014). In the same context, BP-3 did not affect TPO activity in FTC-238/TPO cells and iodide uptake in FRTL-5 cells (Schmutzler et al., 2007b). At the same time, BP-1 and BP-8 activated TPO activity in FTC-238/TPO cell line, which is in opposite direction of BP-2 (Song et al., 2012). According to Schmutzler et al (2007a)., TPO activity depends on H_2O_2 level or I^- level, which indicates exposure condition such as background level of other chemicals could affect whether TPO inhibition would occur.

Considering thyroid hormone-lowering effects of each BP, activation in NIS

may have less contribution to thyroid disruption than inhibition in TPO, as TPO enzyme involves later stages of thyroid hormone synthesis (Zoller et al., 2007). In addition, even though some BPs, such as BP-1 and -8 in this study, highly up-regulated transcriptional level of *Nis*, previous study reported BPs showed no alterations in iodide uptake level. Iodide uptake catalyzed by NIS was not affected by exposure to BP-2 and -3 in FRTL-5 cells in previous studies (Schmutzler et al., 2007a; b).

Following exposure to TSH, a positive chemical in FRTL-5 cells, up-regulation in *Nis* and down-regulation in *Tshr* and *Tg* genes were coincident with the result in Kim et al. (2015) (Fig. 3). However, down-regulation in *Tpo* gene has opposite direction with the previous study: The transcriptional changes in *Tpo* gene following exposure to BPs showed similar direction of change with the previous studies, i.e. inhibition of TPO (Jarry et al., 2004; Schmutzler et al, 2007a;b), which indicated that the result from the FRTL-5 could be valid.

Based on effective concentrations of BPs on T3 alteration in zebrafish larvae, BP-3 and BP-8 appear to be more potent in decreasing thyroid hormones compared to BP-1 (Fig. 5). Previously, EC50 of BP-2 in aspects of intra-follicular T4-content was reported at 4.70 μM (1.16 mg/L) in zebrafish larvae till dpf (Thienpont et al., 2011) which is higher than those observed for other BPs in the present study. . The results of the present study therefore show that some BPs such as BP-3 and -8 might be more potent thyroid disrupting compounds compared to BP-2 in 120 hpf zebrafish. Decreased thyroid hormone levels by all three BPs are in line with previous studies. In

ovariectomized adult rats, after 5 days of oral application of BP-2, significant decrease of serum T4 and T3 levels were reported (Jarry et al., 2004; Schmutzler et al., 2007a). In addition, BP-3 measured in urine showed negative association with free T3 level in serum of pregnant women (Aker et al., 2016) and also with T4 level in serum of general U.S.A. population (Kim et al., 2017).

Decreased thyroid hormone level in zebrafish could result from several reasons. In case of BP-3 and -8, up-regulation in ugt1ab gene could explain decrease in thyroid hormone level. UGT is an enzyme related to excretion of thyroid hormone. Therefore, increased UGT enzyme by exposure to BPs could stimulate excretion of thyroid hormone and result in decreased thyroid hormone level in zebrafish larvae. Also, as reported before, TPO inhibition could accont for decreased level of thyroid hormone level in zebrafish. Compensatory effect could mask inhibition in TPO as shown in the previous study (Schmutzler et al., 2007a). Even though BP-2 disrupts thyroid hormone by inhibiting TPO, stimulating mRNA expression level of *Tpo* gene could be stimulated highly due to low thyroid hormone level in zebrafish.

5. Conclusion

Thyroid disruption potentials were observed from two cell lines and zebrafish larvae for most of BPs tested in the present study. Decreases in thyroid hormone levels in zebrafish model clearly demonstrate thyroid disrupting effects of BP-1, -3, and -8. Considering importance of thyroid hormone regulation in early development and normal physiology, consequences of this thyroid hormone disruption in later life stages warrant further investigation. The results of the present study outline thyroid disrupting effects of BPs which are widely used in consumer products and detected in the environment. Consequences of long-term exposure among humans and ecosystem receptors warrant further studies.

6. References

Aker, A. M., Watkins, D. J., Johns, L. E., Ferguson, K. K., Soldin, O. P., Anzalota Del Toro, L. V., Alshawabkeh, A. N., Cordero, J. F., Meeker, J. D., 2016. Phenols and parabens in relation to reproductive and thyroid hormones in pregnant women. Environmental Research. 151, 30-37.

Baldini, E., D'Armiento, M., Sorrenti, S., Del Sordo, M., Mocini, R., Morrone, S., Gnessi, L., Curcio, F., Ulisse, S., 2013. Effects of ultraviolet radiation on FRTL-5 cell growth and thyroid-specific gene expression. Astrobiology. 13, 536-542.

Blüthgen, N., Zucchi, S., & Fent, K. Baldini, E., D'Armiento, M., Sorrenti, S., Del Sordo, M., Mocini, R., Morrone, S., Gnessi, L., Curcio, F., Ulisse, S., 2013. Effects of ultraviolet radiation on FRTL-5 cell growth and thyroid-specific gene expression. Astrobiology 13, 536-542., 2012. Effects of the UV filter benzophenone-3 (oxybenzone) at low concentrations in zebrafish (*Danio rerio*). Toxicology and Applied Pharmacology. 263(2), 184-94.

Boas, M., Feldt-Rasmussen, U., Skakkebæ k, N. E., Main, K. M., 2006. Environmental chemicals and thyroid function. European Journal of Endocrinology. 154(5), 599-611.

Chang, J., Wang, M., Gui, W., Zhao, Y., Yu, L., Zhu, G., 2012. Changes in thyroid hormone levels during zebrafish development. Zoological Science. 29, 181–4.

Ekpeghere, K. I., Kim, U. J., O, S. H., Kim, H. Y., Oh, J. E., 2016. Distribution and seasonal occurrence of UV filters in rivers and wastewater treatment plants in Korea. Science of the Total Environment. 542, 121-8.

Fent, K., Zenker, A., Rapp, M., 2010. Widespread occurrence of estrogenic UV-filters in aquatic ecosystems in Switzerland. Environmental Pollution. 158(5), 1817-24.

Gao, C. J., Liu, L. Y., Ma, W. L., Zhu, N. Z., Jiang, L., Li, Y. F., Kannan, K., 2015. Benzophenone-type UV filters in urine of Chinese young adults: concentration, source and exposure. Environmental Pollution. 203, 1-6.

Gentilcore, D., Porreca, I., Rizzo, F., Ganbaatar, E., Carchia, E., Mallardo, M., De Felice, M., Ambrosino, C., 2013. Bisphenol A interferes with thyroid specific gene expression. Toxicology. 304, 21-31.

Gutleb, A. C., Meerts, I. A., Bergsma, J. H., Schriks, M., Murk, A. J., 2005. T-Screen as a tool to identify thyroid hormone receptor active compounds. Environmental Toxicology and Pharmacology. 19(2), 231-8.

Hines, E. P., Mendola, P., von Ehrenstein, O. S., Ye, X., Calafat, A. M., Fenton, S. E., 2015. Concentrations of environmental phenols and parabens in milk, urine and serum of lactating North Carolina women. Reproductive Toxicology. 54, 120-8.

Jarry, H., Christoffel, J., Rimoldi, G., Koch, L., Wuttke, W., 2004. Multi-organic endocrine disrupting activity of the UV screen benzophenone 2 (BP2) in ovariectomized adult rats after 5 days treatment. Toxicology. 205(1), 87-93.

Kim, S., Jung, D., Kho, Y., Choi, K., 2014. Effects of benzophenone-3 exposure on endocrine disruption and reproduction of Japanese medaka (*Oryzias latipes*)—A two generation exposure study. Aquatic Toxicology. 155, 244-52.

Kim, S., Jung, J., Lee, I., Jung, D., Youn, H., Choi, K., 2015. Thyroid disruption by triphenyl phosphate, an organophosphate flame retardant, in zebrafish (*Danio rerio*) embryos/larvae, and in GH3 and FRTL-5 cell lines. Aquatic Toxicology. 160, 188-96.

Kim, S., Kim, S., Won, S., Choi, K., 2017. Considering common sources of exposure in association studies-Urinary benzophenone-3 and DEHP metabolites are associated

with altered thyroid hormone balance in the NHANES 2007–2008. Environment International. 107, 25-32.

Kinnberg, K. L., Petersen, G. I., Albrektsen, M., Minghlani, M., Awad, S. M., Holbech, B. F., Holbech, H., 2015. Endocrine-disrupting effect of the ultraviolet filter benzophenone-3 in zebrafish, *Danio rerio*. Environmental Toxicology and Chemistry. 34(12), 2833-40

Krause, M., Klit, A., Blomberg Jensen, M., Søeborg, T., Frederiksen, H., Schlumpf, M., Drzewiecki, K. T., 2012. Sunscreens: are they beneficial for health? An overview of endocrine disrupting properties of UV-filters. International Journal of Andrology. 35(3), 424-36.

Kunz, P. Y., Galicia, H. F., Fent, K., 2006. Comparison of *in vitro* and *in vivo* estrogenic activity of UV filters in fish. Toxicological Sciences. 90(2), 349-61.

Lee, S., Kim, C., Youn, H., & Choi, K., 2017. Thyroid hormone disrupting potentials of bisphenol A and its analogues-*in vitro* comparison study employing rat pituitary (GH3) and thyroid follicular (FRTL-5) cells. Toxicology in Vitro. 40, 297-304.

Liu, C., Wang, Q., Liang, K., Liu, J., Zhou, B., Zhang, X., Liu, H., Giesy, J.P., Yu, H., 2013. Effects of tris (1, 3-dichloro-2-propyl) phosphate and triphenyl phosphate on receptor-associated mRNA expression in zebrafish embryos/larvae. Aquatic Toxicology. 128, 147-157.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{\Delta\Delta CT}$ method. Methods. 25, 402–8.

Ma, B., Lu, G., Liu, F., Nie, Y., Zhang, Z., Li, Y., 2016. Organic UV Filters in the Surface Water of Nanjing, China: Occurrence, Distribution and Ecological Risk Assessment. Bulletin of environmental contamination and toxicology. 96(4), 530-

5.

Miller, M. D., Crofton, K. M., Rice, D. C., Zoeller, R. T., 2009. Thyroid-disrupting chemicals: interpreting upstream biomarkers of adverse outcomes. Environmental health perspectives. 117(7), 1033.

Murk, A. J., Rijntjes, E., Blaauboer, B. J., Clewell, R., Crofton, K. M., Dingemans, M. M., Furlow, J. D., Kavlock, R., Köhrle, J., Opitz, R., Traas, T., Visser, T. J., Xia, M., Gutleb, A. C., 2013. Mechanism-based testing strategy using *in vitro* approaches for identification of thyroid hormone disrupting chemicals. Toxicology In Vitro. 27(4), 1320-46.

Patrick, L., 2009. Thyroid disruption: mechanisms and clinical implications in human health. Alternative Medicine Review. 14(4), 326-47.

Schlecht, C., Klammer, H., Jarry, H., Wuttke, W., 2004. Effects of estradiol, benzophenone-2 and benzophenone-3 on the expression pattern of the estrogen receptors (ER) alpha and beta, the estrogen receptor-related receptor 1 (ERR1) and the aryl hydrocarbon receptor (AhR) in adult ovariectomized rats. Toxicology. 205, 123-30.

Schlumpf, M., Cotton, B., Conscience, M., Haller, V., Steinmann, B., Lichtensteiger, W., 2001. *In vitro* and *in vivo* estrogenicity of UV screens. Environmental Health Perspectives. 109, 239-44.

Schmutzler, C., Bacinski, A., Gotthardt, I., Huhne, K., Ambrugger, P., Klammer, H., Jarry, H., 2007a. The ultraviolet filter benzophenone 2 interferes with the thyroid hormone axis in rats and is a potent *in vitro* inhibitor of human recombinant thyroid peroxidase. Endocrinology. 148(6), 2835-44.

Schmutzler, C., Gotthardt, I., Hofmann, P. J., Radovic, B., Kovacs, G., Stemmler, L.,

Nobis, I., Bacinski, A., Mentrup, B., Ambrugger, P., Gruters, A., Malendowicz, L. K., Christoffel, J., Jarry, H., Seidlova-Wuttke, D., Wuttke, W., and Kohrle, J., 2007b. Endocrine disruptors and the thyroid gland−a combined *in vitro* and *in vivo* analysis of potential new biomarkers. Environmental Health Perspectives. 115, 77−83.

Song, M., Kim, Y. J., Park, Y. K., Ryu, J. C., 2012. Changes in thyroid peroxidase activity in response to various chemicals. Journal of Environmental Monitoring. 14(8), 2121-6.

Suzuki, T., Kitamura, S., Khota, R., Sugihara, K., Fujimoto, N., Ohta, S., 2005. Estrogenic and antiandrogenic activities of 17 benzophenone derivatives used as UV stabilizers and sunscreens. Toxicology and Applied Pharmacology. 203, 9-17.

Thienpont, B., Tingaud-Sequeira, A., Prats, E., Barata, C., Babin, P. J., Raldúa, D., 2011. Zebrafish eleutheroembryos provide a suitable vertebrate model for screening chemicals that impair thyroid hormone synthesis. Environmental Science & Technology. 45(17), 7525-32.

Tsui, M. M., Leung, H. W., Wai, T. C., Yamashita, N., Taniyasu, S., Liu, W., Murphy, M. B., 2014. Occurrence, distribution and ecological risk assessment of multiple classes of UV filters in surface waters from different countries. Water Research. 67, 55-65.

Wang, Q., Liang, K., Liu, J., Yang, L., Guo, Y., Liu, C., Zhou, B., 2013. Exposure of zebrafish embryos/larvae to TDCPP alters concentrations of thyroid hormones and transcriptions of genes involved in the hypothalamic–pituitary–thyroid axis. Aquatic Toxicology. 126, 207-213.

Yen, P. M., 2001. Physiological and molecular basis of thyroid hormone action.

Physiological Reviews. 81(3), 1097-142.

Yu, L., Deng, J., Shi, X., Liu, C., Yu, K., Zhou, B., 2010. Exposure to DE-71 alters thyroid hormone levels and gene transcription in the hypothalamic–pituitary–thyroid axis of zebrafish larvae. Aquatic Toxicology. 97(3), 226-33.

Zoeller, R. T., Tan, S. W., Tyl, R. W., 2007. General background on the hypothalamic-pituitary-thyroid (HPT) axis. Critical Reviews in Toxicology. 37(1-2), 11-53.

Supplementary Information

Table S1. Primer sequences for qRT-PCR analysis used in this study

	Gene	Primer Sequence (5'-3')	Accession no.
GH3	*Gapdh*	F: aacgacccctcattgacct	NM_017008.4
		R : ccccatttgatgttagcggg	
	Tshβ	F: acagaacggtggaaataccg	NM_013116.2
		R: tctgtggcttggtgcagtag	
	Trhr	F: tatcacttgtgagggctgca	NM_031134.2
		R: cacagcgatgcacttcttga	
	Trβ	F: atgttttgtgagctgccctg	J03933.1
		R: catgcccaggtcaaagatcg	
	Dio2	F: cagctttctcctagacgcct	NM_031720.3
		R: gcaaagtcaagaaggtggca	
FRTL-5	*β-actin*	F: tcttccagccttccttcctg	NM_031144.3
		R: cacacagagtacttgcgctc	
	Tshr[a]	F: aggacatggtgtgtaccccc	NM_012888.1
		R: aatctgcaaaggccaggttg	
	Tpo	F: ccacaattgccaacctgtca	NM_019353.2
		R: tgggctgactgaaaccatct	
	Nis	F: tgcaccttgtacactaccgt	NM_052983.2
		R: ccgaggatcagggtcaaagt	
	Tg[b]	F: acgatgggcttatcaacagg	XM_006241707.1
		R: atatggcagcagcaaggatg	
Zebrafish	*18s rrna*[c]	F: acgcgagatggagcaataac	FJ915075
		R: cctcgttgatgggaaacagt	
	tshβ[d]	F: gcagatcctcacttcacctacc	AY135147
		R: gcacaggtttggagcatctca	
	slc5a5	F: ggtggcatgaaggctgtaat	NM_001089391.1
		R: gatacgggatccattgttgg	

tpo	F: gatcatcacccgtctccttc	XM_017351696.1
	R: tcctgctcgacttctccttc	
tg	F: ttgctctgtggttcaaagcc	KU662327.1
	R: agtcggtgttgctcagaaga	
dio1[d]	F: gttcaaacagcttgtcaaggact	BC076008
	R: agcaagcctctcctccaagtt	
dio2	F: ttctccttgcctcctcagtg	NM_212789.3
	R: agccacctccgaacatcttt	
pax8[d]	F: gaagatcgcggagtacaagc	AF072549
	R: ctgcactttagtgcggatga	
ugt1ab[d]	F: ccaccaagtctttccgtgtt	NM_213422
	R: gcagtccttcacaggctttc	

[a] Gentilcore et al. (2013); [b] Baldini et al. (2013); [c] Liu et al. (2013); [d] Wang et al. (2013); primers of which reference was not denoted were designed using Primer 3 online software ver. 4.0.0 (http://primer3.ut.ee/).

Figure S1. Result of preliminary range-finding test of GH3 assay. Cell proliferation levels following exposure to 6 benzophenones (n=3).

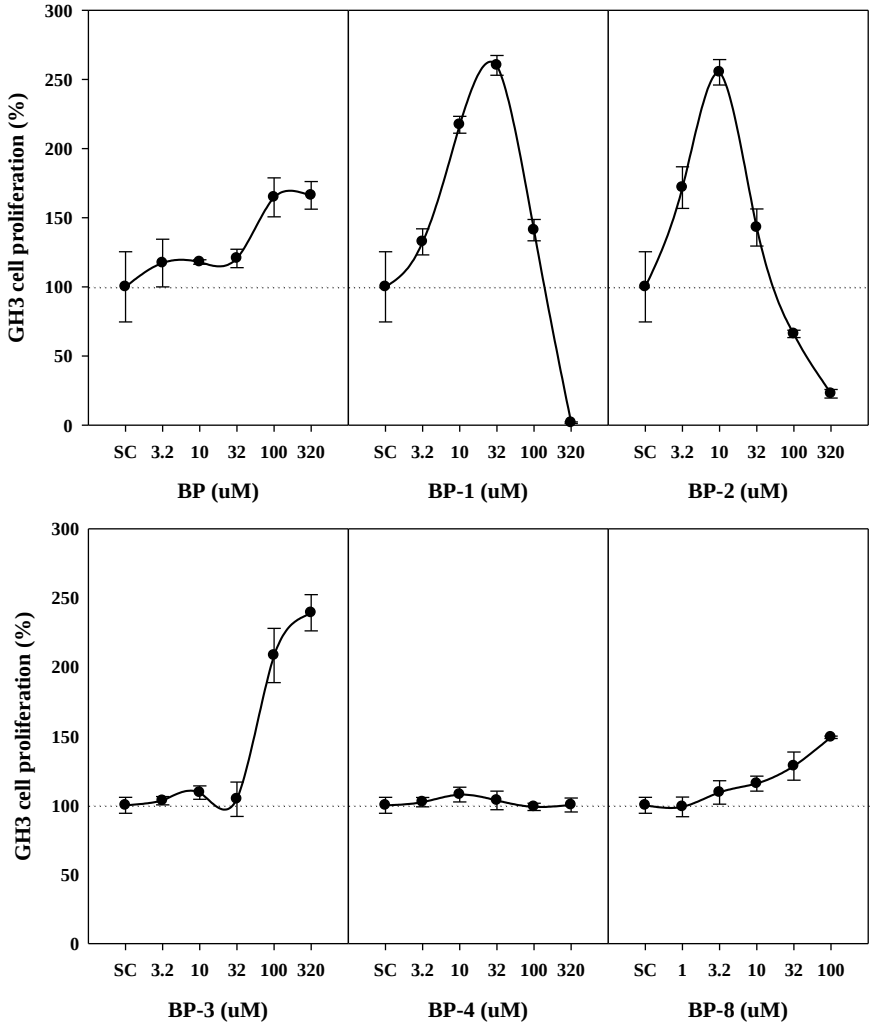
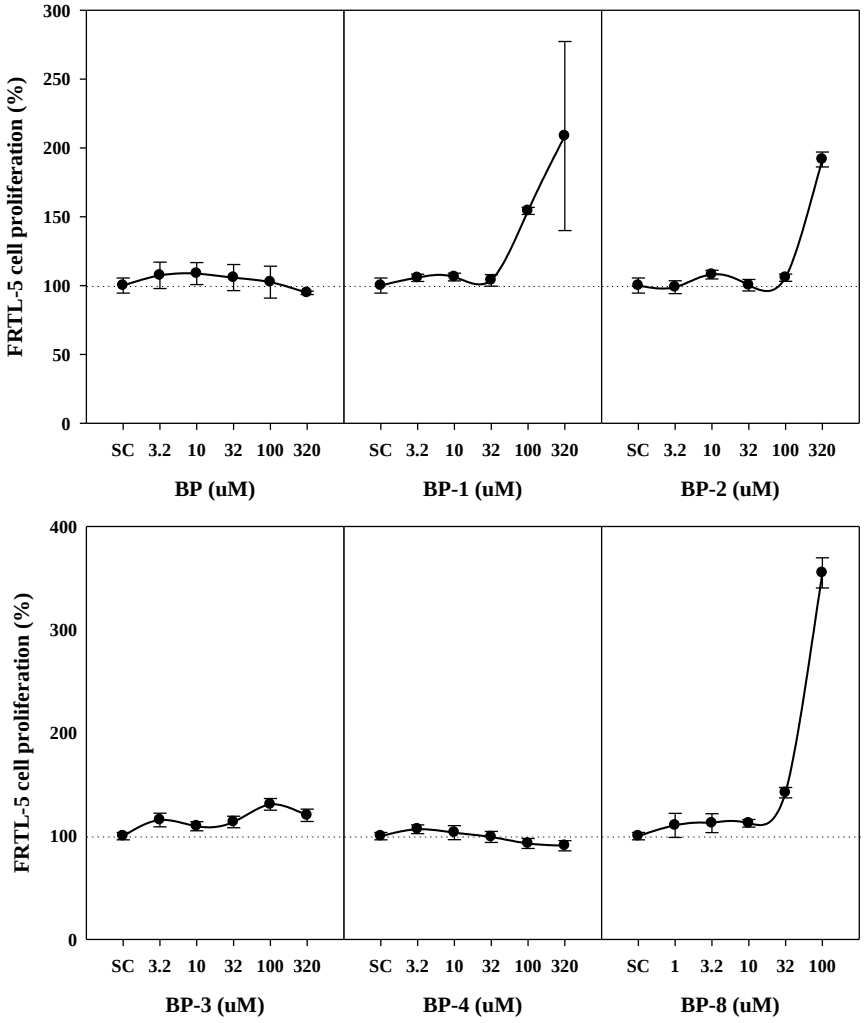


Figure S2. Result of preliminary range-finding test of FRTL-5 assay. Cell proliferation levels following exposure to 6 benzophenones (n=3).



국문 초록

GH3, FRTL-5 세포주와 제브라피쉬를 활용한 벤조페논류의 갑상선 교란 영향 스크리닝

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벤조페논류는 화장품 등의 개인 생활 용품에 포함되어 자외선 차단제로 널리 이용되며 환경 및 인체 시료에서 빈번하게 검출되고 있다. 일부 벤조페논류의 내분비계 교란 영향이 보고된 바 있으나, 그 구조 유사체에 대한 갑상선 교란 영향에 대한 연구는 제한적인 실정이다.

따라서 본 연구에서는 여섯 종의 벤조페논, 즉 벤조페논, 벤조페논-1, -2, -3, -4, 그리고 -8을 대상으로 랫드 뇌하수체 세포주인 GH3 세포와 랫드 갑성선 세포주인 FRTL-5에서 갑상선 관련 유전자의 변화를 관찰하였다. 이어서 자외선 차단제 성분으로 빈번하게 이용되는 BP-3와 그 대사체인 BP-1 및 BP-8의 3종 벤조페논류를 제브라피쉬 수정란에 144시간 동안 노출시켜 갑상선 호르몬 수준 및 관련 유전자 변화를 관찰하였다.

GH3 세포 노출 결과, 벤조페논-4를 제외한 대상 벤조페논류 모두에서 *Tsh*β, *Trhr*, *Tr*β에서의 감소 영향 및 *Dio2*에서의 증가 영향을 나타냈다. FRTL-5 세포

노출 결과, 일부 벤조페논류에서 *Tpo* 유전자의 감소 경향을 나타내었고, *Nis* 및 *Tg* 유전자에서 증가 경향을 나타내었다. 제브라피쉬 노출 후 세가지 벤조페논 모두 T4 및 T3를 유의하게 감소시킴을 관찰하였다. 벤조페논-3와 -8이 벤조페논-1보다 낮은 노출 농도에서도 T3를 감소시켜 두 물질의 갑상선 교란 영향이 벤조페논-1보다 높은 수준임을 시사하였다. 갑상선 호르몬 조절 과정에 기여하는 유전자 역시 함께 변화되었음을 관찰하였다.

본 연구에서 평가된 벤조페논류는 갑상선 시스템의 항상성 조절 기능을 담당하는 뇌하수체에서의 갑상선 교란 영향을 보였다. 제브라피쉬 수정란-치어 노출 역시 벤조페논-1, -3 및 -8가 갑상선 호르몬 수준을 변화시킴을 나타내었다. 갑상선 호르몬은 초기 발달단계와 정상적인 생리 작용에 중요한 기능을 담당하므로, 이러한 갑상선 교란이 발생 이후의 단계에 미치는 영향에 대한 연구가 추가적으로 필요하다.

주요어: 벤조페논, 자외선 차단제, GH3 세포주, FRTL-5 세포주, 제브라피쉬, 갑상선 호르몬, 내분비계 교란, 스크리닝

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